Pathogenesis and Clinical Signs of Equine Herpesvirus-1 in Experimentally Infected Ponies in vivo

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ABSTRACT

Equine herpesvirus-1 (EHV-1) causes respiratory disease, neonatal death, abortion and neurologic disease. The main purpose of this study was to identify viral antigen in respiratory tract samples by immunoperoxidase staining. Six pony foals were selected on the basis of demonstrating seronegativity to EHV-1 by virus neutralization and housed in isolation. They were infected experimentally by administering EHV-1 nebulized ultrasonically through a face mask. Successful infection was clinically apparent as each of the foals had febrile responses, nasal discharge, and enlarged submandibular lymph nodes. Sporadic coughing was also heard. EHV-1 was isolated from nasopharyngeal swabs of 4/6 ponies and seroconversion was demonstrated in all foals. Bronchoscopic examination of the large airways revealed hyperemia. The incidence of recovery of *Actinobacillus suis* from nasopharyngeal swabs increased initially, with recovery of *Streptococcus zooepidemicus* isolates predominating at 3 wk post-infection. Cytology brushes were used to sequentially sample the respiratory tract of the infected ponies at the nasopharynx, mid-trachea and the mainstem bronchus. Bronchoalveolar lavage provided lung cells. Immunocytochemistry techniques were applied to both types of samples to locate EHV-1 antigen. Indirect immunoperoxidase staining of samples utilizing monoclonal antibodies specific for EHV-1 demonstrated viral antigen associated with cellular debris, primarily in the nasopharyngeal samples on days 3–9 post-infection.

RÉSUMÉ

Le virus herpès équin de type 1 (VHE-1) est une cause de maladie respiratoire, de mortinatalité, d'avortement et de problème neurologique. Le but de la présente étude était d'identifier l'antigène viral dans des échantillons provenant du tractus respiratoire au moyen d'une coloration par l'immunoperoxidase. Six poulains séronégatifs au test de neutralisation virale furent sélectionnés, gardés en isolement puis infectés expérimentalement par nébulisation d'une suspension de VHE-1. La réussite de l'infection était apparente cliniquement chez tous les animaux tel que démontré par la réponse fébrile, l'écoulement nasal et l'hypertrophie des ganglions sous-mandibulaires. Une toux sporadique fut également notée. Le virus fut isolé chez 4/6 poulains à partir d'écouvillonnage du nasopharynx et une séroconversion fut démontrée chez tous les poulains. Une hyperémie au niveau des voies respiratoires fut notée lors de l'examen bronchoscopique. L'incidence d'isolement d'*Actinobacillus suis* à partir des prélèvements du nasopharynx a augmenté initialement, suivi, à trois semaines post-infection, d'une prédominance d'isolement de *Streptococcus zooepidemicus*. À l'aide de brosses à cytologie, des échantillons furent prélevés au niveau du nasopharynx, de la mi-trachée et des bronches principales. Un lavage broncho-alvéolaire a permis de recueillir des cellules pulmonaires. Les analyses par immunocytochimie furent effectuées sur les divers prélèvements afin de localiser l'VHE-1. Une coloration des spécimens par immunoperoxidase indirecte à l'aide d'anticorps monoclonaux spécifiques à VHE-1 a permis de démontrer l'antigène viral en association avec des débris cellulaires, principalement dans les échantillons provenant du nasopharynx, entre les jours 3 et 9 post-inoculation.

(Traduit par docteur Serge Messier)

INTRODUCTION

Equine herpesvirus respiratory disease in horses has been traditionally described as a self-limiting infection of young horses (1), caused by either equine herpesvirus-1 (EHV-1) or equine herpesvirus-4 (EHV-4) (2). However, equine herpesvirus-1 infection can also cause neonatal death, abortion and debilitating neurologic disease (2). Latency of both EHV-1 and EHV-4 has been detected by polymerase chain reaction (PCR) (3) and is believed to be widespread in the equine population of the United Kingdom, as demonstrated by PCR and co-cultivation of susceptible cell cultures with tissues collected from abattoir horses by Edington et al (4). Latent virus can experience recrudescence upon stress (5) and result in new outbreaks of respiratory disease.

Advances in the understanding of the pathogenesis of EHV-1 have recently localized viral infection of intranasally infected ponies to the respiratory tract (6) and associated lymphatic tissue (7). These studies were

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carried out using virus isolation and immunohistochemistry on post-mortem tissues from two 7 mo old foals and 12 ponies aged 3–10 y. Other studies have also utilized similar immunohistochemical techniques in order to identify EHV-1 in post-mortem samples (8,9). One recent immunohistochemical study, which identified EHV-1 in aborted fetuses, concluded that immunohistochemistry was comparable to virus isolation for identification of the virus in paraffin-embedded tissue (9). Reagents for immunohistochemical assays are available commercially, with kits being sensitive, specific, and easy to use (10).

The main purpose of this experiment was to sequentially study the distribution of the EHV-1 virus in the airways of 6 experimentally infected animals over time using immunohistochemistry. A secondary purpose was to describe the clinical, bronchoscopic and bacterial flora changes that occur during EHV-1 infection.

MATERIALS AND METHODS

ANIMAL SELECTION AND HOUSING

Six conventional pony foals (5 male; 1 female, mean weight of 129 kg ± 13.1 SD; age range 8–10 mo), from a closed research herd were tested at 2–4 mo of age for antibody to EHV-1 using a virus neutralization assay. Low titres to EHV-1 (≤ 1/64) were identified. Since the herd had not experienced recent respiratory disease or abortion, it was assumed that these titres were due to maternal antibody. Testing was repeated when the foals were 6–8 mo of age, when antibody titres had dropped to undetectable levels as expected (11). General good health status was established by clinical examination, complete blood cell counts and biochemical profiles. At the end of the experiment all the ponies were tested for antibody to equine rhinovirus types 1 and 2, equine viral arteritis, equine herpesvirus-2 and equine adenovirus using virus neutralization assays to rule out any possibility of concurrent viral infections. No seroconversions were noted to any of these viruses.

The ponies were housed in 2 isolated rooms, which provided containment for infectious diseases (Isolation Unit, University of Guelph, Guelph, Ontario), with all Animal Care Code requirements fulfilled. Three ponies were randomly assigned to each room.

AEROSOL CHALLENGE

A local EHV-1 isolate recovered from a horse with respiratory disease and designated #761 (Veterinary Laboratory Services, Ontario Ministry of Agriculture Food and Rural Affairs, Guelph, Ontario) was obtained at the 4th passage level in rabbit kidney cells. This isolate was typed as EHV-1 by indirect immunofluorescence using murine monoclonal antibodies specific to EHV-1 (GP Allen, University of Kentucky, Lexington, Kentucky, USA) (12) in association with FITC-F(ab')2 goat anti-mouse IgG (H+L) (Zymed Laboratories, Inc., So. San Francisco, California, USA). Tests using similar indirect fluorescent antibody assays and monoclonal antibodies specific to EHV-4 (GP Allen, University of Kentucky, Lexington, Kentucky, USA) (12) were negative.

The challenge inoculum was clarified cell culture fluid from the 5th cell culture passage of #761. This inoculum was prepared using equine ovary cells (New York State Diagnostic Laboratory, Cornell University, Ithaca, New York, USA) grown in EMEM (Flow Laboratories, Mississauga, Ontario) supplemented with 2% irradiated fetal bovine sera (CanSera, Rexdale, Ontario) and 5% NuSerum V (Collaborative Research Incorporated, Bedford, Massachusetts, USA). Each pony was exposed to 3.84×10^7 cell culture infective doses ($CCID_{50}$) of virus by aerosolization with an ultrasonic nebulizer (Monaghan Medical Supply, London, Ontario). The nebulizer produces an average particle size of 5 microns which is the ideal size for deposition deep in the lungs (13). A face mask, which fit tightly over the nose of the animal, was attached to the nebulizer via corrugated tubing.

CLINICAL ASSESSMENT

Clinical signs of disease, bronchoscopic evaluation, hematologic changes and plasma fibrinogen levels were monitored over the course of the experiment. The normal reference ranges used were for foals of comparable age (14).

BACTERIOLOGY AND MYCOLOGY

Nasopharyngeal swabs were cultured for bacteria and fungal isolates (Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario) according to standard techniques (15).

VIRUS ISOLATION

The nasopharyngeal swabs used in sampling for virus isolation were made by sewing 1 1/2″ medium cotton rolls of pediatric dental swabs (Richmond Dental Cotton Co., Charlotte, North Carolina, USA) to polyethylene surgical tubes (21″ long) with polyamid suture material (Vetafil gauge 1). This design was intended to maximize the number of cells retrieved during sampling. The rods were inserted into an outer tubing (16″ long with the outer diameter of 1.7 cm). A blind end was fashioned by cutting the sampling end of the outer tube in such a way as to produce a flap which could be tucked back into the tubing. The intended purpose of the blind end was to guard the swab until the nasopharynx was accessed for sampling. During sampling, the guarded swab was introduced into the nasopharynx via the nares. The inner rod was advanced through the blind end of the outer tubing, wedged up against the nasopharyngeal mucosa, rolled to obtain cells from the mucosa and returned into the outer tubing for protection during retraction from the nares. The guarded swabs were used only once and were gas sterilized (ethylene oxide) prior to use. Specimen swabs were frozen at −70°C in virus transport medium (VTM), which consisted of Hank's balanced salts (Flow Laboratories), 0.5% lactoalbumin hydrasolate (Grand Island Biological Co. Chagrin Falls, Ohio, USA), 1% gelatin, 25 mM Hepes, 5000 U/L penicillin, 5000 1g/L streptomycin, and 50 000 U/L mycostatin.

The resulting supernatant was filtered through a 0.45 nm syringe filter (Millipore Products Division, Bedford, Massachusetts, USA), with 0.2 mL inoculated onto decanted equine ovary cell monolayers planted 24 h earlier with 7.7×10^5 equine ovary cells in EMEM supplemented with 10% irradiated fetal bovine sera and 5% NuSerum V. After 1 h incubation at 37°C cell monolayers were rinsed 3 times, refed with EMEM

containing 2% irradiated fetal bovine sera, incubated at 37°C for 5 d and examined daily for cytopathic effect (CPE) for 5 d. Each specimen was passaged 3 times before being considered negative or until CPE typical of herpesviruses was noted (16). Each viral isolate was typed by indirect immunofluorescence as described above with monoclonal antibodies specific to EHV-1.

VIRUS SEROLOGY

Virus neutralization tests for detection of EHV-1 antibody were performed by standard microtitre technique, using embryonic bovine spleen cells and 100 CCID of EHV-1 Kentucky D virus (17). Seroconversion was defined as a change from a negative to a positive antibody titre over the course of the experiment.

IMMUNOCYTOCHEMISTRY SAMPLE COLLECTION

Ponies were tranquilized with intravenous xylazine (0.75 mg/kg) and butorphanol (0.05 mg/kg), before a fibreoptic bronchoscope (GIF type P2, Length 100 cm Olympus gastroscope — external diameter of 8 mm; Carsen Medical and Scientific Co. Ltd., Markham, Ontario) was introduced via 1 naris. Cytology brushes within polyethylene tubing (brush diameter 1.7 mm; length 140 cm; BJ Medical Ltd., Oakville, Ontario) were wetted with sterile phosphate buffered saline solution (PBS) and introduced into the biopsy channel of the bronchoscope. The brushes were exteriorized and the mucosa was sampled. Each brush was used once daily and cold sterilized nightly (Sporicidin International, 12 000 Old Georgetown Road, Rockville, Maryland, USA). Bronchoalveolar lavage (BAL) specimens were collected as described by Viel (18), except that only 120–180 mL of warm PBS was infused due to the smaller size of the pony foals. The mean percent of volume returned on bronchoalveolar lavage was 57.9% ± 14.8 SD. The nasopharynx, mid-trachea, and right mainstem bronchus were sampled by cytology brush on days 0, 3, 5, 7, 9, 11, 13 and 21 post-infection (PI) and BAL samples were collected on days 0, 3, 7, 11 and 21 PI.

The BAL fluid volumes were centrifuged (5 min at 700 × *g*) and the pellets collected. These BAL pellets were crush smeared, while brush samples were directly smeared onto microscope slides. Both were air dried quickly, fixed immediately in cold acetone for 10 min (19) and then stored at −70°C. The endoscope was cleaned and cold sterilized between ponies.

IMMUNOCYTOCHEMISTRY PROTOCOL

The primary antibody for immunoperoxidase (IP) staining was the same murine monoclonal antibodies specific for EHV-1 as used for immunofluorescence staining. A streptavidin-biotin indirect immunoperoxidase assay (Zymed Histostain-SP assay, Dimensions Laboratory, Mississauga, Ontario), prepared for use with primary antibodies prepared in mice was used. The chromogen provided in the assay was aminoethyl carbazole, which resulted in a reddish-brown specific stain. The IP staining proceeded according to manufacturer's instructions with the following modifications. The fixed slides were warmed to room temperature and rehydrated by immersing in a bath of fluorescent antibody (FA) buffer solution (Bacto FA buffer pH = 7.2, Wheaton Scientific, Brampton, Ontario) for 25 min. In order to minimize background staining for samples collected with the cytology brushes, a modified washing solution was prepared. The detergent Tween 20 (Harleco Diagnostics, BDH Chemicals, Toronto, Ontario), was added to the FA buffer described above at a concentration of 0.01% (v:v) (20). The washing solution was applied dropwise to the smears and allowed to incubate at room temperature for 10 min. This additional incubation step was incorporated after the samples were rehydrated and before the primary antibody was applied. After this additional step, the primary antibody was applied at the optimal dilution and incubated overnight (16–19 h) at 4°C. This modified washing solution was used to wash the cytology brushing slides between the IP staining steps. For the BAL smears, the FA buffer used for washing between IP staining steps was not modified, since the Tween 20 detergent removed the BAL cells from the slides.

The endogenous peroxidase block recommended by the manufacturer was applied after the primary antibody incubation in order to maximize antibody recognition of antigen. In order to prevent drying during the primary antibody incubation and the chromogen incubation steps, large coverslips (22 mm × 60 No. 1, Corning, Fisher Scientific Canada, Nepean, Ontario) were applied for the appropriate time periods and the slides were sealed in a wet chamber.

IMMUNOCYTOCHEMICAL CONTROLS

Positive control slides for immunoperoxidase staining were prepared by infecting Madin-Darby bovine kidney (MDBK) cells, grown on glass coverslips with EHV-1 (Army-183, Dr J. Thorsen, Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario). MDBK cells were grown in EMEM supplemented with 2% fetal bovine sera, 0.1% lactoalbumen hydrolysate, 200 U/mL penicillin, and 80 μg/mL streptomycin. Infected cell monolayers were recognized by visual evidence of cytopathic effect and were fixed in acetone as for samples above. Further confirmation of cell culture infection with EHV-1 was established by immunofluorescent staining with monoclonal antibodies in the indirect fluorescent antibody assay, similarly as for identification of the challenge inoculum.

For immunocytochemical staining known infected and uninfected cell culture monolayers and specimens from experimental subjects were stained using monoclonal antibody specific for EHV-1. Duplicate cell cultures and specimen slides were stained with a negative control antisera, which consisted of normal mouse serum since normal murine ascitic fluid was not available. This negative control mouse serum was diluted three times more than the monoclonal antibodies in order to adjust for differences in total immunoglobulin concentration.

Since blood cells contain endogenous peroxidase, a blood smear was included with each group of specimens being stained, in order to demonstrate that the peroxidase block was successful. To be sure that the enzyme and the substrate were functioning properly, the enzyme conjugate and substrate-chromogen solutions were combined and the intensity

TABLE I. Mean values (± SEM) of hematology and differential cell counts in EHV-1 infected ponies on various days post-infection

DPI	WBC	NEUTR	LYMPH	MONO	PLATELET	FIBRINOGEN
0	10.6 ± 0.5	5.2 ± 0.6	4.7 ± 0.4	0.27 ± 0.07	312.3 ± 26.4	3.017 ± 0.22
3	8.0 ± 0.5*	3.7 ± 0.8	4.1 ± 0.3	0.01 ± 0.01*	217.0 ± 18.2*	4.367 ± 0.17*
5	8.3 ± 0.5	3.3 ± 0.5*	4.3 ± 0.2	0.22 ± 0.06	219.0 ± 27.0*	4.250 ± 0.20*
7	10.5 ± 0.8	3.9 ± 0.5	5.4 ± 0.4	0.49 ± 0.1	291.0 ± 22.7	3.667 ± 0.28*
9	10.7 ± 0.7	4.7 ± 0.8	5.2 ± 0.4	0.32 ± 0.08	271.3 ± 17.9	3.367 ± 0.12
11	12.7 ± 1.0	7.4 ± 1.2*	6.2 ± 0.9	0.19 ± 0.05	262.8 ± 25.5*	3.633 ± 0.20*
13	12.7 ± 0.8	6.6 ± 0.7	5.5 ± 0.3	0.22 ± 0.08	343.1 ± 32.4	3.017 ± 0.15
21	12.8 ± 0.9	6.1 ± 0.8	5.4 ± 0.8	0.16 ± 0.05	263.2 ± 28.0	2.738 ± 0.84

units of cell counts = 10^9/L; units of FIBRINOGEN = g/L; * = $P < 0.05$ as compared to Day 0 PI; DPI = day post-infection; WBC = total white blood cell count; NEUTR = neutrophil count; LYMPH = lymphocyte count; MONO = monocyte count; PLATELETS = platelet count

TABLE II. Bacterial culture results for nasopharyngeal swabs taken from EHV-1 infected ponies on days post-infection. The number of foals in which relatively large numbers of colonies were cultured/number of foal samples cultured are recorded

Microorganism	Day 0	Day 3	Day 5	Day 13	Day 21
B. bronchiseptica	6/6	4/6	2/6	1/6	4/6
A. suis	1/6	2/6	2/6	5/6	1/6
β-*Streptococcus*	—	1/6	—	—	—
S. zooepidemicus	—	—	1/6	1/6	6/6
I-Streptococcus	—	—	1/6	—	—
A. equuli	—	—	—	—	1/6
A. lignierisi	—	—	—	—	2/6

B. = Bordetella; S. = Streptococcus; A. = Actinobacillus

of the brick red colour was visually assessed.

CRITERIA FOR POSITIVE IMMUNOCYTOCHEMISTRY STAINING

Positive staining of infected MDCK cell culture monolayers was primarily located in swollen and syncytial cells on the periphery of focal areas of cell loss.

Samples taken from the respiratory tract of experimental subjects were considered adequate when there were greater than 20 cells present per slide. A viral antigen positive sample was defined as a sample processed with the primary antibody specific for EHV-1, in which at least 3 areas of reddish-brown stain precipitin appeared in the form of large amorphous granules, apparently associated with cellular debris. Any non-granular, homogeneous stain unassociated with cells, cellular debris or stain in the form of crystals was discounted. If any reddish-brown staining appeared in the duplicate sample, that was stained with the control negative mouse serum as the primary antiserum, both samples were considered uninterpretable, and the results considered negative (10). As well any reddish-brown staining that occurred along the edge was similarly discounted, since wiping or drying damage may have been responsible for stain precipitation. All samples were read in a manner blind as to the location of sampling, the sampling day and the pony identification.

STATISTICS

The sign test, a non-parametric statistical method was used to evaluate changes in hematological and clinical parameters in comparison to the day of infection (Statistix, Analytical Software, St. Paul, Minnesota, USA).

RESULTS

CLINICAL ASSESSMENT

All the ponies developed pyrexia which ranged from 39.1°C–41.0°C on days 1 and 2 post-infection. Mucopurulent nasal discharge was noted in all ponies by days 4 and 5 and lasted until day 10. Sporadic coughing was heard, but was not a salient feature of the disease. Enlarged submandibular lymph nodes were noted in all 6 foals from days 3–4 until day 10. No dyspnea, abnormal lung sounds or chest pain was noted in any of the ponies.

Bronchoscopic findings included hyperemia of the nasopharynx and the cranial portion of the tracheal mucosae, as well as a purulent mucous present in the trachea. The hyperemia of the nasopharyngeal and tracheal mucosae began as linear streaks of red on day 3 PI. The red streaks subsequently became blotchy and irregular, before fading away completely by day 11 PI. On day 21 PI, 2 of the 6 ponies still showed hyperemia of the mainstem bronchus. Purulent mucous was noted in the trachea of most foals on days 5 and 7 PI. There was very little edema of the nasopharyngeal mucosa and little change in the pharyngeal lymphoid follicles.

There was a statistically significant ($P < 0.05$) drop in total white blood cells on day 3 PI compared to day 0. This appeared to be primarily due to a neutropenia (Table I). Monocyte levels also dropped significantly ($P < 0.05$) on day 3 compared to day 0, but the absolute values of both neutrophils and monocytes were considered within normal limits for foals in this age group. A neutrophilic leucocytosis, which was above the published normal range (14), occurred on days 11, 13 and 21 PI (Table I). This was deemed a statistically significant change ($P < 0.05$) in comparison to pre-infection values. The fibrinogen levels remained within normal published reference ranges, but were statistically elevated on days 3, 5, 7 and 11 PI (Table I) ($P < 0.05$) in comparison to levels noted on day 0.

BACTERIOLOGY AND MYCOLOGY

Fungal isolates were sporadic with *Mucor* spp. and *Aspergillus fumigatus* recovered on 3 occasions. Over the 1st 2 wk there was a trend towards an increasing number of isolates of *Actinobacillus suis*, with a decreasing number of isolates of *Bordetella bronchiseptica*. Isolates of *B. bronchiseptica* and *A. suis* returned to normal incidences by day 21, while the incidence of *Streptococcus zooepidemicus* increased at that time (Table II).

VIRUS ISOLATION

EHV-1 was primarily isolated on days 3 and 5 PI (Table III). Two ponies failed to shed detectable levels of virus at any time. All isolates were identified as EHV-1 using the indirect fluorescent antibody technique.

VIRAL SEROLOGY

All ponies seroconverted to EHV-1, with titres ranging from 1:6 to 1:64 by 21 days PI (Table III).

IMMUNOCYTOCHEMISTRY STAINING

Immunoperoxidase staining of control uninfected and infected MDBK cell cultures was consistent and reliable, with no false positives or false negative tests noted. All of the foals showed evidence of infection by immunoperoxidase staining, since every foal demonstrated at least 2 samples in which there were 3 large amorphous granules of reddish-brown stain, apparently associated with cellular debris. Intact cells demonstrating viral antigen were not found, such that intracellular localization of viral antigen was not possible. EHV-1 antigen was mostly identified in the nasopharynx (17 times), although it was detectable at all the other sites at various times PI (6 times) (Table IV). Virus was demonstrated in the nasopharynx of at least 1 pony on every monitoring day. Four of 6 ponies demonstrated viral antigen in the nasopharynx on day 5 PI. In addition 2 of the prechallenge nasopharyngeal samples were interpreted as containing viral antigen (Table IV).

TABLE III. EHV-1 virus isolation results and virus neutralization titres from experimentally infected foals at days post-infection

	Foal								Day post-infection serology		
#	0	3	5	7	9	11	13	21	0	14	21
3	–	+	+	–	–	–	–	–	< 1:2	<1:2	1:24
4	–	–	+	–	–	+	–	–	< 1:2	1:6	1:64
16	–	–	–	–	–	–	–	–	< 1:2	1:8	1:16
1	–	+	+	–	–	–	–	–	< 1:2	1:8	1:48
10	–	–	+	–	–	–	–	–	< 1:2	1:12	1:16
14	–	–	–	–	–	–	–	–	< 1:2	< 1:2	1:6

TABLE IV. Immunoperoxidase (IP) staining results — Number of positive samples detected at each sampling site on each sampling day/ the number of samples included in the analysis. Included, for comparison, are the number of ponies in which EHV-1 was isolated from the nasopharynx on each day PI

Day PI	0	3	5	7	9	11	13	21
VI	—	2/6	4/6	—	—	1/6	—	—
IP Staining								
nasopharynx	2/6	2/5	4/6	2/6	2/6	1/6	2/6	2/5
trachea	—	—	1/6	1/6	—	—	—	—
bronchus	—	—	1/6	—	—	—	1/6	1/6
BAL	—	1/6	N/D	—	N/D	—	N/D	—

PI = post-infection; VI = virus isolation; BAL = bronchoalveolar lavage; N/D = not done

DISCUSSION

This study was unique as a pathogenesis study of EHV-1 infection in that the same experimental animals were sequentially sampled, rather than being killed at various intervals PI. Since the pathogenesis may not be identical in individual animals this is a distinct advantage, increasing sample sizes for days post-infection. This is beneficial since sample sizes in equine studies are usually very small due to the costs involved in acquiring naive animals and for isolation facilities.

Successful respiratory infection was demonstrated, with clinical signs of disease apparent in all ponies. These clinical signs included elevations in rectal temperature, nasal discharge, enlargement of the submandibular lymph nodes and sporadic coughing. These findings are consistent with most EHV-1 experimental infections recorded in the literature (1,11,21,22). The signs were similar to primary inoculation in naive specific pathogen free (SPF) foal infections (21,23), except that fever resolved sooner. In contrast, SPF foals repeatedly inoculated were reported to have no clinical signs other than a late onset of fever in one report (21) and a shorter period of virus isolation than in this study (22,23).

EHV-1 was isolated from the nasopharynx of 4 of the 6 ponies, with seroconversion to EHV-1 demonstrated in all ponies. The antibody titres were not high when the experiment ended at 21 days PI(£ 1:64). However similar experimental infections with EHV-1, producing clinical signs with virus shedding, have previously recorded a lack of a strong serological responses (24). It is noteworthy that the ponies from which virus was not isolated, showed lower antibody titres.

This is the first detailed report of sequential bronchoscopic findings in the same individuals over the course of experimental EHV-1 infection. The bronchoscopic findings of linear streaks of hyperaemia in the nasopharynx and trachea may be manifestations of "pharyngitis and tracheobronchitis" (1), "focal necrosis" (2) and "hyperemic and congested nasal mucosa" (11) described previously. Nasopharyngeal mucosal erosions were reportedly noted in a recent pathogenesis study (6) which included histologic examination. The period of time hyperemia was identified in this study (day 3 PI to day 11 PI), although not identical, is comparable to the erosions noted from days 1 to 7 PI in a previously reported study (6).

A neutropenic leucopenia was noted in the 1st wk of infection, and a neutrophilic leucocytosis in the 2nd wk of infection. A subsequent neutrophilia was not reported in recent challenge experiments of specific pathogen-free foals (22,24), and may indicate secondary bacterial infection or other stimuli of inflammatory cells in these conventional foals. Lymphopenia, as described previously (2,22), was not noted in this study, perhaps due to a less severe infection resulting from decreased virulence of the challenge virus following 5 passages in cell culture.

Thrombocytopenia on days 3 and 5 PI was similar to that noted with the neurologic form of EHV-1 (25). It has been postulated that this thrombocytopenia is due to non-specific inflammation (26). There was an additional significant drop in thrombocytes (platelets) on day 11 PI, which is interesting in view of recent findings that suggest a 2nd phase of viral activity occurs around 10 to 14 days PI (27). This suggestion of a 2nd phase of viremia is further supported by the recovery of EHV-1 on day 11 PI from the nasopharynx of one of the ponies in this experiment.

Monocyte cell depletions as a consequence of EHV-1 infection are also unrecorded in the literature. Mason et al (28) report early monocytosis in

2 Thoroughbred horses in training with confirmed EHV-1 infections, which is in contrast to the monocytopenia seen in this study.

Differences in the fibrinogen calculation method used in this study (based on thrombin time) (29) and the one used in the reference article (by modified heat precipitation) (14) may explain why fibrinogen levels were within normal range, but were elevated compared to day 0. Alternatively, the foals assumed normal in the reference study, may have undergone subclinical infections.

The change in the bacterial microflora from predominantly *Bordetella bronchoseptica* to *Actinobacillus suis* by day 14 PI and subsequently to primarily *Streptococcus zooepidemicus* by 3 wk PI, implicates the latter as opportunistic bacterial pathogens.

It was an unexpected finding that the samples collected from the infected ponies did not contain intact cells demonstrating viral antigen, but only cellular debris associated with EHV-1 antigen. The infected cells may have lysed either spontaneously due to the lytic action of EHV-1 (30) or during collection and processing. A similar study by us, utilizing the same sample collection technique in ponies infected with equine influenza virus, did not yield lysed cells (31), suggesting that the lysis was more likely due to EHV-1 infection of the cells.

The nasopharynx was by far the optimal sampling site for demonstration of EHV-1 antigen by IP. There were more EHV-1 antigen positive samples from the nasopharynx than from the trachea, bronchus and bronchoalveolar samples combined. This was not likely to be a consequence of the method of infection, since BAL samples demonstrated viral antigen most commonly when the same challenge method was used with equine influenza infected ponies (31). The nasal turbinates and the soft palate, the most common sites of viral identification in a previous study (6), were not sampled in this experiment. In our study viral antigen was identified most commonly in the 1st wk of infection (days 3 to 9 PI), with ponies not sampled on days 1 and 2 PI. These findings are comparable to those reported earlier (days 1 to 7 PI) (6).

Alveolar macrophages were expected to contain viral antigen, since EHV-1 has been isolated from bronchoalveolar macrophages for other herpesvirus infections, such as in pseudorabies infected swine (32). As well EHV-1 virus has been detected from pulmonary lavage cells of 1/5 ponies 10 wk post-infection by polymerase chain reaction and cocultivation of explanted lymphoid tissue (3). Even though stringent controls and criteria were used for the immunoperoxidase staining in this study, bronchoalveolar lavage samples were difficult to evaluate, with all reddish-brown precipitin in the BALs appearing the same, whether in slides stained with immune serum or non-immune serum. Therefore, results had to be regarded as uninterpretable and specimens were considered negative in all but 1 sample.

For the two prechallenge nasopharyngeal samples from 2 ponies that were interpreted as positive using immunohistochemistry, a number of explanations are possible. The tests could be presumed to be false positives. Alternatively since these 2 ponies were not specific pathogen free foals, the stress of moving to the isolation unit 1 wk prior to sampling might have induced EHV-1 recrudescence at around the time of challenge. However this is unlikely since reinfection at a time 3 mo or less after primary infection does not usually cause clinical disease (21,23). The foals were only 8–10 mo old and would be unlikely to have been recently infected without serological evidence of infection. Although prior EHV-4 infection would not prevent the appearance of clinical signs (33), cross-reaction with EHV-4 is unlikely since the monoclonal antibodies used in the IP tests do not cross-react with EHV-4. The most likely explanation is that the experimental design was the reason why the 2 prechallenge samples were positive. Since ponies infected on the same day were housed together, pre-infection samples from the 3rd foals to be serially infected in each room would have been collected 6 h after the 1st ponies were aerosolized in the same room. At the time the experiment was designed it was presumed that viral antigen could not be detectable in the nasopharynx of adjacent ponies within so short a time. However in a recent EHV-1 experimental infection study EHV-1 was recovered from 1 foal and 1 adult pony 12 h after challenge (6). Virus was isolated from the pharynx and soft palate of the foal and from the soft palate and main bronchus of the adult (the nasopharynx was not sampled from the adult). Peroxidase positive epithelial cells were noted as well (6). The fact that antigen was also demonstrated frequently by immunohistochemistry in the bronchial lymph nodes and occasionally in lymphocytes and dendritic cells of the upper respiratory tract lymph nodes indicated that infection, as well as exposure, had occurred by 12 h post-infection (7). Therefore the 2 ponies in this study, which had immunoperoxidase staining in nasopharyngeal samples collected prechallenge, may have been exposed and infected with EHV-1 due to the experimental design.

In summary, since these methods were minimally invasive, they allowed repeated sampling of the same experimental subjects, providing more samples for evaluation on each sampling day. The experimental animals in this study were young, hence the pathogenesis in naive subjects could be evaluated. This report describes previously unrecorded clinical aspects of EHV-1 experimental infection, including descriptions of sequential bronchoscopic examination and sequential changes in bacterial flora. Immunoperoxidase staining was applied to respiratory cells and cellular debris collected sequentially with cytology brushes and by bronchoalveolar lavage of EHV-1 infected foals. Viral antigen was most commonly identified in the nasopharynx, implicating the nasopharynx as a major site of EHV-1 replication. EHV-1 antigen became detectable in the upper airways within hours of exposure. Viral antigen appeared disassociated from intact cells probably due to cell lysis as a direct result of the viral infection. There appeared to be support for a second phase of viral activity. Future sequential studies should include earlier nasopharyngeal and bronchoalveolar lavage sampling, as well as testing of buffy coat cells and lymph node aspirates.

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